FROM GENE TO PROTEIN – CLONNING, EXPRESSION AND PUFICATION OF A P450 CYTOCHROM FROM *Campylobacter jejuni*

DE LA GENA LA PROTEINA – CLONAREA, EXPRESIA SI PURIFICAREA UNUI CITOCROM P450 DIN *Campylobacter jejuni*

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Recently, the complete genome sequence of Campylobacter jejuni NCTC 11168 was published revealing the presence of only one open reading frame (Cj1411c) encoding for a cytochrome P450, in contrast to 20 found in M. tuberculosis. The gene Cj1411c encodes for a soluble 52.6 kDa protein with a predicted isoelectric point of 9.3. The P450 gene is part of reading frame which hosts genes involved in the synthesis of cell surface components (capsula). Campylobacter capsule are important in adherence, invasion and colonisation of host cells and for maintenance of cell surface charge and serum resistance. These capsule are thought to cause autoimmunity leading to Guillan-Barre and Miller-Fischer syndromes. The structure of the lipoolygosaccharides and capsule polysaccharide was published last year revealing that the strain possessed a type II/III capsule locus found in other microorganisms such Nisseria meningitidis. This project focuses on the cloning and characterisation of the only P450 enzyme of the human pathogen Campylobacter jejuni NCTC 11168. We aim to understand the metabolic role of this P450 cytochrome in order to elucidate its possible use as a new target for drug design. To achieve this aim we have cloned, expressed and purify the product of P450 coding gene.

Keywords: cytochrome P450, cloning, expression, purification

Introduction

The *Campylobacter jejuni 11168* P450 is unique among the P450 enzymes by its unusual location, no other enzymes are known to be located in an operon involved in cell surface biosynthesis (Teramoto, 2004; Kelly, 2001). His closest homologues were identified in *Polaromonas* sp. (38 % identity) and *Silicobacter* sp.(34 % identitiy). *Silicibacter* sp. TM1040 is a member of the *Roseobacter* of the alpha-proteobacteria, which is among the most abundant and ecologically relevant marine bacterial groups. *Polaromonas* strain JS666 (ATCC No. BAA-500), a member of the family *Comamonadaceae* in the beta-proteobacteria, is a novel,

aerobic, *cis*-dichloroethene (cDCE)-assimilating organism with optimum growth at 20-25¾C. Strain JS666 is closely related to the Antarctic marine isolate *Polaromonas vacuolata* (Allos, 1995; Altschul, 1997; Andersen, 1993; Anzenbacher, 2001). Both of these genes are involved in carbohydrate metabolism (ascorbate and aldarate) (Allos, 1997; Bacon 2001).The sequence alignment of these two enzymes with the *Campylobacter jejuni* P450 is presented in figure 4.The cytochrome P450 coding sequence (Cj1411c) of *Campylobacter jejuni 11168* is 1359 bp long. Is located in an open reading frame with an unusual number of genes (40 genes), from which 55 % of the genes involved in sugar or polysaccharide biosynthesis. The remaining 45 % is represented by unknown proteins and few amino acid transferases. In the primary structure the protein contains 453 amino acids having at the C-terminal the characteristic protoporpyrin IX binding motif. In secondary structure the protein is dominated by helixces with few coils and strands.Mitochondrial and bacterial P450 systems have three components: and FAD-containing flavoprotein (NADPH or NADH-dependant reductase), an iron-sulfur protein and P450. By comparison with the P450 Rhf isolated from *Rhodococcus* sp. NCIMB 9784, where the reductase domain is an FMN-FeS domain fused to the N-terminal heme-containing oxygenase, in *Campylobacter jejuni 11168* the reductase components are not fused in a single polypeptide. The FMN was identified under Cj1382c and Fe-S under Cj1377c. Having all the components separate the *Campylobacter* P450 reductase is represented under a new class (class V – figure 1).

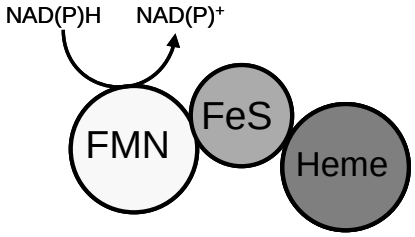

Fig. 1. Diagram of the possible class V of P450 reductases.

The aim of this work was to clone, express and purify the only bacterial cytochrome P450 located in a genomic locus which is charcaterised by the presence of genes involved in sugar and surface structures biosynthesis.

Material and Methods

For cloning and expression of the cytochrome P450 a system constructed by Novagen UK called Perfectly Blunt Cloning Kits (technical details presented across the chapter) which are designed for cloning of any DNA fragment which has 5' overhangs and 3' overhangs was used. The DNA fragments are made blunt-ended by a single step reaction followed by a heat inactivation step and then the fragments are ligated in to the dephosphorylated vector. The recombinant plasmid

is transformed in *E. coli* NOVA BLUE and positive colonies are identified by blue / white screening (pETBlue-1 Blunt vector Manual, NOVAGEN). The pETBlue™ Perfectly Blunt Cloning Kits include *Tuner*™(DE3)placI competent cells for expression. This strain carries a chromosomal copy of the T7 RNA polymerase gene. The expression of the target proteins is controlled by the T7*lac* promoter which is IPTG inducible. The strain NOVA BLUE is used like a mother strain which allows us to check the constructs. The system is suitable for inserts which have an ATG start codon.

Results and Discussion

For genomic DNA preparation *Campylobacter jejunui NCTC 11168* strain was grown on Mueller-Hinton Broth for 42 hours in an atmosphere containing 5 % O_2, 10 % CO_2 and 85 % N_2 in a microaerobic box. Cells were harvested by centrifugation when they reached the stationary phase and genomic DNA was extracted using Wizard Genomic Kit from Promega. P450 gene was amplified from genomic DNA by PCR using the primers Cj1411cFOR and Cj1411cREV. Amplification was made using Promega *PFU* polymerase to avoid any possible mutation during PCR. The PCR reaction was performed using 5pmol of each primer and 1.25 U of *PFU* polymerase. The total volume of reaction was 50 µl

PCR steps:

Step I: 95 ^{0}C for 1 minute - 1 cycle

Step II: 95 ^{0}C for 30 seconds
53.2 ^{0}C for 30 seconds } 32 cycles
72 ^{0}C for 2 minutes

Step III: 72 ^{0}C for 5 minutes - 1 cycle

After amplification each reaction mix was checked in 0.8 % agarose gel and in each case a fragment of the expected size (1359 bp) was observed (fig. 2). PCR reactions were stored at -20 ^{0}C until the next cloning step was performed.

After gel purification the fragment was ligated in pETBlue-1 Blunt vector. The purified insert was cloned into the *EcoRV* site which is located such that an insert will specify the N terminus of the expressed protein.

After ligation the transformation was performed with E. coli Nova Blue competent cells. After transformation, cells were plated on LB solid media supplemented with carbenicilin (50 µg / ml) and tetracycline (15 µg / ml).

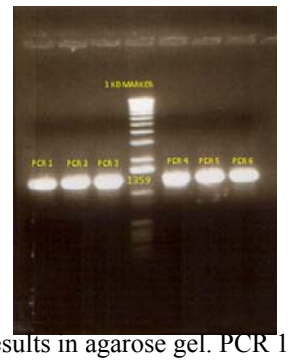

Fig. 2. Visualisation of PCR results in agarose gel. PCR 1,2 and 3 have be done by using 1 µl genomic DNA and 0.5 µl for PCR 4, 5, and 6. 3 µl from each reaction have been used in the gel.

Transformation was performed as described in material and methods. For blue/white screening X-gal and IPTG were added to the media, prior to plating the cells and the plates were incubated overnight at 37 ^{0}C. There were no mutations detected in the sequence. Using the same protocol, but using the primers Cj1411cFOR and Cj1411cREVHIS, we also constructed a gene encoding a recombinant protein with a 6 HIS Tag at the C terminus of the sequence. The construct was cloned into the pETBlue1 Blunt vector and expressed in *E. coli Tuner (DE3) placI*. The plasmid has been named pBP450CjCHIS. For expression the recombinant plasmid pBP450Cj was used to transformed Tuner (DE3) placI. Cells resistant to carbenicilin (50 µg / ml) and chloramphenicol (34 µg / ml) have been used for protein expression. Expression trials were performed in 100 ml flasks containing LB + 1 % glucose and the appropriate antibiotics which were inoculated with 1 ml of o/n culture. Cultures were than incubated at 37 ^{0}C by shaking at 200 rpm until the cultures has reached 0.5 O.D. For expression cells were induced with 1 mM IPTG and samples were taken at different intervals for testing the protein expression. For protein expression tests 1 ml of cell culture was centrifuged and the pellet diluted in 50 µl water. The pellet was boiled for 5 minutes and 2 µl of solution was mixed with 5 µl SDS PAGE loading buffer and water up to 15 µl. Samples were run in 15 % SDS PAGE gel and the proteins visualised by coomassie staining (fig. 3). The red coloured pellet characteristic of P450 expression appeared after 24 hours. The native enzyme was identified by spectral characterisation and CO binding reaction. A 450 nm peak was observed in whole cell extracts (fig. 4).

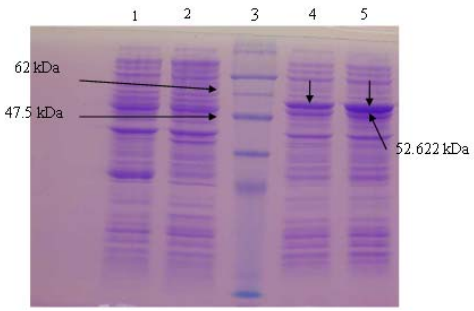

Fig. 3. Visualisation of protein expression using 15 % SDS-PAGE and stained with Coomassie Blue. Lane 1 – cells only (no plasmid), Lane 2 – uninduced (no IPTG), Lane 3 – marker, Lane 4 – expression after 8 hours, Lane 5 – expression after 24 hours

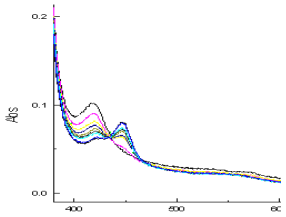

Fig. 4. Expression identified by 450 nm absorption in SORET band with CO bound. Sample preparation: cell membranes were disrupted by sonication, centrifuged and supernatant collected. To reduce the protein sodium dithionite was used and CO was bubbled into the sample. Successive spectra shows the transition of the 420 nm peak, of the oxidised protein, to 450 nm peak by exposure of the protein to the CO.

The initial purification trials were started with the anion exchange resins DEAE Sephacel and Q sepharose and with hydroxyapatite, using TRIS-HCl buffer, HEPES (pH 6.8-8.2), Phosphate buffer (pH 6-8) and CAPS (pH 9.7-11). Unfortunately the protein did not bind to the resins in any of these conditions. The same problems were faced with the cation exchangers C25 and CM (carboxymethyl cellulose) at pH 7 the protein came through the column without any binding. The next attempts were to try the hydrophobic resins: phenyl sepharose, octyl sepharose, decyl sepharose, butyl sepharose. None of these columns provided a good purification. The protein was bound to the phenyl sepharose column but it could not be eluted. For purification, eight flasks containing 1.5 L LB, 1 % glucose and the appropriate antibiotics (see materials and methods) were inoculated with 5 ml of Tuner (DE3)placI containing the plasmid pBP450Cj. Cultures were grown at 37 ^{0}C for ~ 6 hours or until the cultures reached 0.5 O.D. by shaking at 230 rpm. Cells were induced for protein expression with 1 mM IPTG and incubated for another 24 hours at 18 ^{0}C. After 24 hours cells were harvested in 0.5 L bottles in a RC-5B centrifuge at 8000 rpm and the pellet was washed with 20 mM triethanolamine - HCl at pH 8 and centrifuged again for

collection. Cells were again resuspended in 20 mM triethanolamine - HCl at pH 8 and cells sonicated as described in materials and methods. Cell lysate was centrifuged for 30 minutes at 15000 rpm in a RC-5B centrifuge and the supernatant kept. The supernatant was than treated with 40 mg RNaseA and 0.9 mg DNaseI and was gently mixed for 35 minutes at room temperature. The pellet was than centrifuged again for 30 minutes at 15000 rpm in a RC-5B centrifuge. In the next step the supernatant was treated with protamine sulphate (200 mg / l culture) and mixed occasionally for 15 minutes at room temperature. Then the pellet was centrifuged at 11200 rpm for 30 minutes at 4 ^{0}C and the supernatant retained. The supernatant was frozen at –20 ^{0}C or used directly in the purification steps and a small volume was used to check the activity. Purification of the P450 enzyme was done using a DE52 resin by using the following protocol:(i) after treating with RNaseA, DNaseI and protamine sulphate the crude cell extract was loaded into a 100 ml column containing DE52 resin equilibrated with 300 ml 20 mM triethanolamine – HCl, pH 8. The P450 protein is coming through the column and a part other proteins from the solution binds to the resin. (ii) the red fraction was concentrated and dialysed o/n to change the buffer to ml 20 mM, triethanolamine – HCl, pH 8 and 200 mM KCl and was loaded into a DE52 column equilibrated in the same buffer. (iii) to achieve a better purification the protein was loaded into the third DE52 column. All the fractions were verified by SDS-PAGE and stained with Coomassie blue (fig.5).

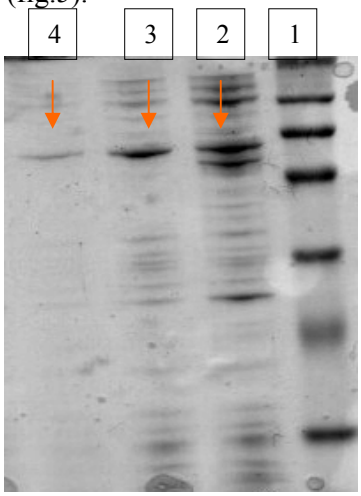

Fig. 5. Purification on DE52 – fractions visualisation on 15 % SDS-PAGE and Coomassie Blue Staining:

1. First DE52 after RNaseA, DNaseI and protamine sulphate treatment
2. Second DE52
3. Third DE52

The protein concentration was approximated to 12.5 mg / ml and was calculated using the SORET absorption. The concentrated protein was frozen at – 80 ^{0}C until further experiments were preceded.

Conclusions

This paper describes the work carried out on the cytochrome P450 enzyme from *Campylobacter jejuni 11168*. Sequencing in 2000 of the *Campylobacter*

jejuni 11168 genome revealed the presence of a cytochrome P450 coding sequence which was proved to be unique by its localisation in an operon involved in cell surface biosynthesis. This cytochrome P450 was identified to be conserved among the *Campylobacter jejuni* strains by its presence in *Campylobacter jejuni RM1221* (Aspinall, 1994, 1993). The recombinant protein was successfully over-expressed using pETBlue1 Blunt as vector and *E. coli* Tuner (DE3) placI as expression strain, this study being a very useful tool for expression of recombinant P450s. Purification of this cytochrome P450 was not completely successful but 70 % purity was achieved using DE52 anion-exchange and triethanolamine as a buffer at pH 8. Purification was done in reverse way by collecting the red fraction which ran through the column. The red fraction collected was concentrated up to 12 mg/ml but at this concentration the protein proved to be cloudy and very viscous. To improve the purity of the protein to HIS-Tagged constructs were prepared (one with the 6HIS at the N terminus and one at the C terminus) but the purification trials failed as the protein was not bound to the nickel column. By comparison with the P450 Rhf isolated from *Rhodococcus* sp. NCIMB 9784, where the reductase domain is an FMN-FeS domain fused to the N-terminal heme-containing oxygenase, in *Campylobacter jejuni 11168* the reductase components are not fused in a single polypeptide. The FMN was identified under Cj1382c and Fe-S under Cj1377c. Having all the components separate the *Campylobacter* P450 reductase is represented under a new class. Being the only cytochrome P450 known to be involved in sugar metabolism future work must be done on purification and in trying to obtain a crystal structure which would be a major step helping to understand the interaction with electron transfer partners. Biochemical studies must be developed on kinetics of the enzyme giving insights in the function of this novel enzyme and to identify the substrate and the product. On the biological side further experiments must be conducted to study the difference in adhesion and infectivity between the wild type strain and a P450 deficient strain. Because of its location in a reading frame in which all the genes are involved in surface structure biosynthesis it is very important to investigate the phenotypic behaviour of a P450 deficient strain. The results obtained following these experiments could be very important for the pharmaceutical industry that is looking for new targets in creating an efficient vaccine against *Campylobacter* spp.

References

1. Allos, B.M., 1997, Association between *Campylobacter* infection and Guillian-Barre syndrome. *J. Infect. Disease*. **176**(S2), S125-S128.
2. Allos, B.M., Blaser, M.J., 1995. *Campylobacter jejuni* and the expanding spectrum of related infections. *Clinical Inf. Disease*, **20**, 1092-1101.
3. Altschul, St. F., Madden, T., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W.. and Lipman, D. J., 1997, "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res*. **25**:3389-3402.

4. Andersen, J.F. *et al.*, 1993, Substrate specificity of 6-deoxyerythronolide B hydroxylase, a bacterial cytochrome P450 of erythromycin A biosynthesis. *Biochemistry* **32**, 1905–1913.
5. Anzenbacher, P., Anzenbacherova, E., 2001, Cytochromes P450 and metabolism of xenobiotics, *Cell. Mol. Life Sci.* **58**, 737–747.
6. Aspinall GO, McDonald AG, Pang H, Kurjanczyk LA, Penner JL.1994, Lipopolysaccharides of *Campylobacter jejuni* serotype O:19: structures of core oligosaccharide regions from the serostrain and two bacterial isolates from patients with the Guillain-Barre syndrome, *Biochemistry*. **33**(1), 241-9.
7. Aspinall GO, McDonald AG, Raju TS, Pang H, Moran AP, Penner JL., 1993, Chemical structures of the core regions of *Campylobacter jejuni* serotypes O:1, O:4, O:23, and O:36 lipopolysaccharides. *Eur J Biochem*. **216**(3):880.
8. Bacon, D.J., Szymanski, C.M., Burr, D.H., Silver, R.P., Alm, R.A., Guerry, P. 2001, A phase-variable capsule is involved in virulence of *Campylobacter jejuni* 81-176. *Mol Microbiol*. **40**(3):769-77.
9. De Mot, R., Parret, A.H., 2002, A novel class of self-sufficient cytochrome P450 monooxygenases in prokaryotes. *Trends Microbiol*. **10**(11), 502-8
10. Hiroshi Teramoto, Hiroo Tanaka and Hiroyuki Wariishi, 2004, Fungal cytochrome P450s catalyzing hydroxylation of substituted toluenes to form their hydroxymethyl derivatives, *FEMS Microbiology Letters,* **234**, Issue 2, 255-260
11. Kelly, D.J., 2001, The physiology and metabolism of *Campylobacter jejuni* and *Helicobacter pylori*. *Journal of Applied Microbiology* **90**, 16S-24S.